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Complexes of different nitrogen donor heterocyclic ligands with $SbCl_3$ and $PhSbCl_2$ as potential antileishmanial agents against Sb^{III}-sensitive and -resistant parasites

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ABSTRACT

Novel trivalent antimony complexes with the nitrogen donor heterocyclic ligand 2,2′-bipyridine (bipy), 1,10-phenanthroline (phen) or dipyrido[3,2-d:2′,3′-f]quinoxaline (dpq) have been synthesized by the reaction with $SbCl_3$ or $PhSbCl_2$. The crystal structures of $[Sb(phen)Cl_3]$ and $[PhSb(phen)Cl_2]CH_3COOH$ were determined and shown to adopt a distorted square pyramid geometry with a five-coordinated Sb center. Surprisingly, all the complexes, the ligands and $PhSbCl_2$ showed very high antileishmanial activities, with IC_{50} in the nanomolar range against Sb^{III}-sensitive and -resistant *Leishmania infantum* (syn. *Leishmania chagasi*) and *Leishmania amazonensis* strains. These compounds were much more active against these *Leishmania* strains than the old trivalent drug potassium antimonyl tartrate. $[PhSb(phen)Cl_2]CH_3COOH$ complex was found to be the most active compound and the lack of cross-resistance of $PhSbCl_2$ suggests that the transport pathways of this compound across the cell membrane differ from those responsible for the resistance of *Leishmania* to $Sb(OH)_3$. In the case of the complexes with $PhSbCl_2$, our data supports the model that both ligand and metal contributed to the overall activity of the complex. Furthermore, among the complexes with $SbCl_3$, only bipy showed an improved activity upon complexation. Cytotoxicity evaluations of these compounds against murine peritoneal macrophages showed high selective indexes in the range of 7–70 for $[Sb(phen)Cl_3]$, $[Sb(bipy)Cl_3]$ and $[Sb(dpq)Cl_3]$ complexes, being much more selective than potassium antimonyl tartrate. In conclusion, this study presents a set of new antileishmanial agents including one of the most active Sb-based compounds ever reported, which can contribute to the development of new chemotherapeutic strategies against leishmaniasis including Sb-resistant cases.

1. Introduction

Pentavalent antimonials such as sodium stibogluconate and meglumine antimoniate, have been used in the treatment of all forms of leishmaniasis for more than half a century [1,2]. Although the mechanism of action of pentavalent antimonial is not fully understood, it is generally accepted that the active form of the metal is the reduced form Sb^{III} [3,4].

A major problem in antimonial chemotherapy is the emergence of clinical resistance against pentavalent antimony drugs that has reached epidemic proportions in parts of India [5]. The ATP binding cassette (ABC) protein MRPA plays a major role in metal resistance in *Leishmania* parasites [6] and its localization in intracellular vesicle membranes suggests that it sequesters Sb^{III}-thiol complexes into these vesicles [7]. Other mechanisms such as a diminished biological reduction of Sb^{V} to Sb^{III} [8], the loss of an aquaglyceroporin (AQP1) allele or its down regulation [9,10] and hypoxic conditions [11] have been reported to cause an increase in resistance to pentavalent antimonials. In this context, there is a great need for new safe and effective drugs that do not exhibit cross-resistance with conventional antimonial drugs.

It was suggested that the mode of action of trivalent antimonial compounds involves some pathways similar to apoptosis, as DNA fragmentation [12,13], which is preceded by an increase in reactive oxygen species caused by alterations of the redox potential [14]. As an antileishmanial agent, Sb^{III} inhibits and forms a complex with the enzyme trypanothionereductase which acts by recycling trypanothione disulfides in trypanothione, the major antioxidant thiol in *Leishmania* [15,16]. Furthermore, zinc finger domains in proteins, which were associated with protein–nucleic acid interactions [17,18], were recently proposed as potential targets for Sb^{III}, due to the ability of Sb^{III} to compete with Zn^{II} for its binding to the CCHC and CCCH zinc finger domains [19,20].

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In the search for safer and more effective Sb^{III} complexes, our group recently investigated dipyrido[3,2-a:2′,3′-c]phenazine (dppz) as a polypyridyl ligand [21]. The resulting antimony complex showed a high antileishmanial activity in the micromolar range, but the metal did not seem to be active by itself. It was proposed that complexation decreased the lipophilicity of dppz, resulting in enhanced dppz activity.

As a continuation, the present study investigates for the first time the antileishmanial activity of Sb^{III} complexes with three other nitrogen donor heterocyclic ligands: a less lipophilic polypyridyl ligand dipyrido [3,2-d:2′,3′-f]quinoxaline (dpq), 2,2′-bipyridine (bipy) and 1,10-phenanthroline (phen). $PhSbCl_2$ was also evaluated as a new active form of Sb^{III}, as an attempt to enhance the biological activity of the metal complex.

It is noteworthy that phen was shown to exhibit antileishmanial activity against *Leishmania braziliensis* and *Leishmania amazonensis* [22,23], which was attributed to its ability to inhibit *Leishmania* proteinases through chelation of Zn ion [23–27]. More recently, ternary copper(II) as well as oxidovanadium(IV) complexes bearing phen ligand were also presented as promising antileishmanial compounds [28,29].

The present work reports the synthesis, the structural and physicochemical characterization of the complexes between the nitrogen donor heterocyclic ligands and $SbCl_3$ or $PhSbCl_2$. The ligands (dpq, bipy and phen), $SbCl_3$, $PhSbCl_2$ and their resulting complexes were tested against both Sb^{III}-sensitive and -resistant *Leishmania infantum* (syn. *Leishmania chagasi*) and *L. amazonensis* promastigotes. The cytotoxicity of these compounds towards murine peritoneal macrophages is also reported.

2. Experimental

2.1. Materials

phen, bipy, Sb^{III} chloride ($SbCl_3$), $SbPh_3$ and potassium antimonyl(III) tartrate were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Dipyrido[3,2-d:2′,3′-f]quinoxaline (dpq) was synthesized according to Liang et al. [30].

Phenyl antimony(III) dichloride ($PhSbCl_2$) was prepared as described previously [31] by reorganization of 1/2 molar mixtures of $SbPh_3$ and $SbCl_3$ at 25 °C. The solid $PhSbCl_2$ was isolated from the resulting viscous liquid through crystallization from glacial acetic acid. All the preparations were done under anhydrous conditions.

2.2. Synthesis of the complexes

A solution of $SbCl_3$ or $PhSbCl_2$ (0.2 mmol) dissolved in chloroform (10 mL) was added dropwise to a chloroform solution (15 mL) containing 0.2 mmol of polypyridyl ligand (2,2′-bipyridine, 1,10-phenanthroline or dipyrido[3,2-d:2′,3′-f]quinoxaline). The mixture was stirred in reflux system for 1 h at room temperature. Then the mixture was filtered, the precipitate was collected, washed with 20 mL of fresh chloroform and then dried *in vacuo*. The phen complexes were recrystallized in DMSO.

2.3. Physicochemical characterizations

Elemental analyses for C, H and N were carried out using a Perkin Elmer 2400 CHNS/O elemental analyzer. IR spectra in the 4000–400 cm^{-1} region were obtained from KBr pellets using the Perkin Elmer RX-83303 FTIR spectrophotometer.

Conductivity of the complexes was measured in DMSO at 10^{-3} M using DM31 Digimed apparatus equipped with a conductivity cell (1.185 cm^{-1}). All measurements were carried out at room temperature with freshly prepared solutions.

NMR spectra were recorded at 200 MHz using Bruker DPX-200 spectrometer. ^1H and ^{13}C NMR chemical shifts were measured relative to tetramethylsilane (TMS) and dimethyl sulfoxide-d6 (DMSO-*d*6) as the solvent. Intensity data for the X-ray were collected with Xcalibur Atlas Gemini ultra. Crystallographic data and experimental details of the structure determinations are listed in Table 1.

Table 1
Crystal data and structure refinement parameters for $[Sb(phen)Cl_3]$ and $[PhSb(phen)Cl_2]CH_3COOH$.

Complex	$[Sb(phen)Cl_3]$	$[PhSb(phen)Cl_2]CH_3COOH$
Chemical formula	$C_{12}H_8Cl_3N_2Sb$	$C_{22}H_{19}Cl_2N_2O_2Sb$
Formula weight	408.30	510.03
Temperature (K)	293 (2)	150 (2)
Crystal system	Triclínic	Monoclínic
Space group	P-1	C2/c
a (Å)	8.4284 (2)	19.4337 (17)
b (Å)	8.5966 (2)	13.8280 (4)
c (Å)	9.9016 (3)	18.9136 (13)
α (o)	82.401 (2)	90
β (o)	72.928 (3)	129.481 (12)
γ (o)	76.798 (2)	90
V ($Å^3$)	666.05 (3)	3923.0 (5)
Z	2	4
F (000)	392	2016
Dcalc (mg/m3)	2.036	1.727
Crystal dimensions mm^3	0.38 × 0.38 × 0.15	0.30 × 0.29 × 0.09
θ Range (°)	2.16–32.82	2.00–29.5
Reflections collected	25,514	10,400
Independent reflection	4680	4686
R_{int}	0.0344	0.025
Maximum/minimum transmission	1.000/0.706	1.000/0.742
Data/restraints/parameters	4680/0/163	4680/0/246
Goodness-of-fit on F^2	1.131	1.190
Final *R* indices [I > 2σ(I)]	*R1* = 0.0254, w*R2* = 0.064	*R1* = 0.0335, w*R2* = 0.0935
R indices (all data)	*R1* = 0.0354, w*R2* = 0.0644	*R1* = 0.0458, w*R2* = 0.1085
Largest difference in peak/hole (e·$Å^{-3}$)	0.5 and −0.79	1.53 and −0.81

The stability of the complexes in aqueous solution was checked by UV absorption spectroscopy. For this purpose, the complexes and their respective ligand were diluted at 40 μM in phosphate-buffered saline (PBS: 0.15 M KCl, 0.05 M phosphate, pH 7.0) from 4 mM stock solutions in DMSO. UV spectra were registered just after dilution and after 4-h of incubation at 25 °C. The spectra of the complexes were different from those of their respective ligands and did not suffer significant change after 4-h of incubation (see Supplementary materials). This data indicates that the complexes do not readily dissociate upon dilution in aqueous solution.

2.3.1. [Sb(bipy)Cl_3]

The bipy complex was characterized by infrared spectra, ^1H NMR, ^{13}C NMR and elemental analysis. Yield: 87% (67.0 mg). IR (KBr, cm^{-1}): 1600, 1584, 1526 ν(C═C, C═N); 760 δ(Csp^2-H). ^1H NMR (DMSO-d_6 400 MHz) δ: 8.72 (d, $H_{6,9}$, J = 4 Hz); 8.41 (d, $H_{3,12}$, J = 8 Hz); 7.99 (t, $H_{4,11}$, J = 8Hz); and 7.49 (t, $H_{5,10}$, J = 6 Hz). ^{13}C NMR (DMSO-d_6) δ: 120.83 ($C_{3,12}$), 124.58 ($C_{5,10}$), 137.98 ($C_{4,11}$), 148.95 ($C_{6,9}$), and 154.30 ($C_{2,7}$). Elemental analysis for $C_{10}H_8Cl_3N_2Sb$, calc. (found): C% 31.25 (31.24); H% 2.10 (2.04); and N% 7.29 (7.60).

2.3.2. [Sb(phen)Cl_3]

The phen complex was characterized by infrared spectra, ^1H NMR, ^{13}C NMR, elemental analysis and X-ray diffraction. Yield: 88% (71.5 mg). IR (KBr, cm^{-1}): 1574, 1516, 1424 ν(C═C, C═N); 716 δ(Csp^2-H). ^1H NMR (DMSO-d_6 400 MHz) δ: 9.60 (dd, $H_{1,10}$, J = 4 Hz); 8.96 (dd, $H_{3,8}$, J = 8 Hz); 8.29 (s, $H_{5,6}$); and 8.18 (dd, $H_{2,9}$, J = 8 Hz). ^{13}C NMR (DMSO-d_6) δ: 125.72 ($C_{2,9}$), 127.62 ($C_{5,6}$), 129.72 ($C_{4,7}$), 138.60 ($C_{3,8}$), 141.32 ($C_{11,12}$), and 147.75 ($C_{1,10}$). Elemental analysis for $C_{12}H_8Cl_3N_2Sb$, calc. (found): C% 35.30 (35.67); H% 1.97 (1.98); and N% 6.86 (6.96).

2.3.3. [PhSb(bipy)Cl_2]

The bipy complex was characterized by infrared spectra, ^{1}H NMR, ^{13}C NMR, and elemental analysis. Yield: 85% (72.0 mg). IR (KBr, cm^{-1}): 1597, 1592, 1528 ν(C═C, C═N); 764 δ(Csp^2-H). ^{1}H NMR (DMSO-d_6 400 MHz) δ: 8.72 (d, $H_{6,9}$, J = 4 Hz); 8.42 (d, $H_{3,12}$, J = 8 Hz); 7.94–8.08 (m, $H_{14,18,4,11}$); and 7.51–7.28 (m, $H_{5,10,15,17,16}$). ^{13}C NMR (DMSO-d_6) δ: 120.59 ($C_{3,12}$), 124.35 ($C_{5,10}$), 127.57 ($C_{15,17}$), 128.44 (C_{16}), 134.57 ($C_{14,18}$), 137.56 ($C_{4,11}$), 149.17 ($C_{6,9}$), 154.89 (C_{13}), and 160.19 ($C_{2,7}$). Elemental analysis for $C_{16}H_{13}Cl_2N_2Sb$, calc. (found): C% 45.12 (44.34); H% 3.08 (3.10); and N% 6.58 (6.64).

2.3.4. [PhSb(phen)Cl_2]CH_3COOH

The phen complex was characterized by infrared spectra, ^{1}H NMR,^{13}C NMR, elemental analysis and X-ray diffraction. Yield: 86% (87.4 mg). IR (KBr, cm^{-1}): 1576, 1518, 1428 ν(C═C, C═N); 724 δ(Csp^2-H). ^{1}H NMR (DMSO-d_6 400 MHz) δ: 11.92 (s, OH); 9.38 (dd, $H_{1,12}$, J = 4 Hz); 8.81 (d, $H_{3,10}$, J = 12 Hz); 8.18 (s, $H_{6,7}$); 8.09–8.02 (m, $H_{22,26}$ and $H_{2,11}$); 7.33–7.16 (m, H_{24} and $H_{23,25}$); and 1.91 (s, H_{31}). ^{13}C NMR (DMSO-d_6) δ: 125.01 ($C_{2,11}$), 127.22 ($C_{23,25}$), 127.35 ($C_{6,7}$), 127.84 (C_{24}), 129.34 ($C_{4,8}$), 135.10 ($C_{22,26}$), 139.93 ($C_{3,10}$), 140.54 ($C_{5,9}$), 148.54 ($C_{1,12}$), and 161.13 (C_{21}). Elemental analysis for $C_{20}H_{17}Cl_2N_2O_2Sb$, calc. (found): C% 47.10 (47.14); H% 3.36 (3.20); and N% 5.49 (5.42).

2.3.5. [Sb(dpq)Cl_3]

The dpq complex was characterized by infrared spectra, ^{1}H NMR, ^{13}C NMR and elemental analysis and. Yield: 77% (71.0 mg). IR (KBr, cm^{-1}): 1620, 1582, 1536 ν(C═C, C═N); 1124 δ(Csp^2-H). ^{1}H NMR (DMSO-d_6 400 MHz) δ: 9.72 (d, $H_{7,16}$, 8 Hz); 9.37 (d, $H_{5,18}$, 4 Hz); 9.30 (s, $H_{11,12}$); and 8.29 (t, $H_{6,17}$, 6 Hz). ^{13}C NMR (DMSO-d_6) δ: 126.60 ($C_{6,17}$), 128.02 ($C_{8,15}$), 136.99 ($C_{7,16}$), 138.79 ($C_{9,14}$), 140.69 ($C_{2,3}$), 146.67 ($C_{11,12}$), and 149.45 ($C_{5,18}$). Elemental analysis for $C_{14}H_8Cl_3N_4Sb$, calc. (found): C% 36.52 (36.10); H% 1.75 (2.00); and N% 12.17 (12.10).

2.4. X-ray crystallography

Single crystal X-ray diffraction data were obtained at room temperature on a Xcalibur Atlas Gemini Ultra diffractometer, using graphite mono chromate MoKα radiation (λ = 0.71069 Å). Final unit cell parameters and the integration of the collected reflections were performed using the CRYSALISPRO software (version 1.171.33.55 release 05-01-2010 CrysAlis171.NET). The structure solutions and full-matrix least-squares refinements based on *F*2 were performed with the SHELXS-97 and SHELXL-97 program package [32]. All atoms except hydrogen were refined anisotropically. Although many hydrogen atoms could be identified in a Fourier difference map all of them were geometrically added to the structure and then refined by the riding model in the final stages. Details of data collection and structure refinement are given in Table 2. Selected distances and angles are given in Table 3. The crystallographic data were deposited at the Cambridge Crystallographic Data Centre (CCDC 946085 and 946086—www.ccdc.cam.ac.uk).

Table 2
Selected bond length (Å) and angles (°) for complex [Sb(phen)Cl_3].

Ligation	Distance(Å)	Angle	(°)
Sb(1)–Cl(1)	2.5805 (6)	Cl(3)–Sb(1)–Cl(1)	161.38 (2)
Sb(1)–Cl(2)	2.5013 (6)	Cl(2)–Sb(1)–Cl(1)	86.87 (2)
Sb(1)–Cl(3)	2.5493 (7)	Cl(2)–Sb(1)–Cl(3)	95.61 (3)
Sb(1)–N(1)	2.3963 (17)	N(1)–Sb(1)–Cl(3)	89.78 (5)
N(1)–C(1)	1.332 (3)	N(2)–Sb(1)–Cl(3)	80.01 (5)
N(1)–C(12)	1.355 (2)	N(1)–Sb(1)–Cl(2)	155.13 (4)
Sb(1)–N(2)	2.2408 (16)	N(2)–Sb(1)–Cl(2)	85.97 (4)
N(2)–C(10)	1.334 (2)	N(1)–Sb(1)–Cl(1)	80.69 (4)
C(11)–N(2)	1.364 (2)	N(2)–Sb(1)–Cl(1)	81.76 (5)
		N(2)–Sb(1)–N(1)	71.05 (6)

Table 3
Selected bond length (Å) and angles (°) for complex [PhSb(phen)Cl_2]CH_3COOH.

Ligation	Distance(Å)	Angle	(°)
Sb(1)–Cl(1)	2.6168 (9)	C(21)–Sb(1)–Cl(1)	87.37 (9)
Sb(1)–Cl(2)	2.5723 (10)	Cl(2)–Sb(1)–Cl(1)	104.78 (3)
Sb(1)–N(1)	2.418 (3)	N(1)–Sb(1)–Cl(1)	162.33 (7)
Sb(1)–N(2)	2.377 (3)	N(2)–Sb(1)–Cl(1)	94.41 (7)
Sb(1)–C(21)	2.178 (4)	C(21)–Sb(1)–Cl(2)	88.52 (10)
N(1)–C(1)	1.323 (4)	N(1)–Sb(1)–Cl(2)	90.47 (7)
N(1)–C(5)	1.366 (4)	N(2)–Sb(1)–Cl(2)	158.64 (8)
N(2)–C(9)	1.370 (4)	C(21)–Sb(1)–N(1)	84.12 (11)
N(2)–C(12)	1.328 (4)	C21–Sb1–N2	83.00 (11)
		N(2)–Sb(1)–N(1)	69.23 (9)

2.5. In vitro antileishmanial assay

2.5.1. Parasite strains

L. (*Leishmania*) *amazonensis* (strains MHOM/BR/1989/BA199, sensitive and resistant to Sb^{III} at concentration up to 2700 μM) and *L.* (*Leishmania*) *infantum* (syn. *L. chagasi*) (strains MCAN/BR/2002/BH400, sensitive and resistant to Sb^{III} at concentration up to 2700 μM) promastigotes were maintained in minimum essential culture medium (alpha-MEM) (Gibco, Invitrogen NY, USA) supplemented with 10% (v/v) heat inactivated fetal calf serum (FBS, Cultilab, Campinas, SP, Brazil), 100 mg/mL kanamycin, 50 mg/mL ampicillin, 2 mM L-glutamine, 5 mg/mL hemin, 5 mM biopterin (Sigma-Aldrich, St Louis, USA), at pH 7.0 and incubated at 25 °C. *L. amazonensis* and *L. infantum* (syn. *L. chagasi*) were selected for Sb^{III} resistance as described previously [33]. The Sb^{III}-resistant mutants *L. amazonensis* BA199 Sb^{III} 2700.2 and *L. infantum* (syn. *L. chagasi*) BH400 Sb^{III} 2700.2 were selected in 25 cm^2 flasks containing 5 mL of alpha-MEM in the presence of increasing Sb^{III} concentrations up to 2700 μM. The concentration that inhibits the parasite growth by 50% (IC_{50}) was calculated by non-linear regression using the GraphPad Prism 5 software.

2.5.2. Antileishmanial activity

Compounds were evaluated in vitro for their activity against both Sb^{III}-sensitive and -resistant *Leishmania* parasites, as described previously [21]. Briefly, log-phase *L. amazonensis* and *L. infantum* (syn. *L. chagasi*) promastigotes (1×10^6 parasites/mL) were seeded in 24-well cell culture plates with 1.5 mL of alpha-MEM. The cells were incubated under shaking at 25 °C for 72 h in the presence of seven different concentrations of the compound to be tested. Non-treated parasites were established for growth comparison. Stock solutions of the drugs were prepared in DMSO and diluted in alpha-MEM cell culture medium to obtain the range of tested concentrations. The final DMSO concentration did not exceed 0.2%, which is known to be nontoxic to *Leishmania* parasites [34]. For drug susceptibility assays, *Leishmania* growth curves were constructed by measuring absorbance at 600 nm. Antileishmanial activity is expressed as IC_{50}/72 h, which is the compound concentration that reduces cell growth by 50% compared to untreated control (relative growth). All experiments were performed in triplicate.

2.6. Cytotoxicity assay against murine peritoneal macrophages

The cytotoxicity of the compounds towards murine peritoneal macrophages was evaluated using the classical 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) method [35]. Briefly, macrophages were obtained by lavage of the peritoneal cavities of Swiss mice with 10 mL cold RPMI-1640 (Gibco, Invitrogen NY, USA) without FBS. After washing, the cell suspension (4.0×10^6/mL) was seeded (0.1 mL) in 96-well flat bottom plates. Macrophages were allowed to adhere for 1 h at 37 °C and non-adherent cells were removed by washing with RPMI. The compounds to be studied were then added to the wells at varying concentrations and the cells were further cultured in RPMI supplemented with 10% FBS for 24 h at 38 °C in a humidified 5%

CO_2 atmosphere. Thereafter, the medium was replaced with fresh RPMI containing 0.5 mg/mL of MTT and the plates were incubated for an additional 4 h. Supernatants were aspirated, and the formazan crystals formed were dissolved in 100 μL of DMSO. After 15 min of incubation at room temperature, the absorbance of solubilized MTT formazan product was measured spectrophotometrically at 570 nm. Cell viability was calculated from the ratio of the absorbance of the well treated with the drug to that of the non-treated well. The concentration of drug that decreased cell viability by 50% (CC_{50}) was determined.

3. Results and discussion

3.1. Synthesis and characterization of antimony complexes

The Sb^{III} complexes were prepared by direct reaction of ligands (bipy, phen or dpq) with 1:1 mole ratio of $SbCl_3$ or $PhSbCl_2$ in chloroform under inert conditions. The conductivity of the complexes in DMSO (10^{-3} M) at room temperature (13.70–27.00 μS/cm) showed that they are non-electrolytes. The elemental analysis of $[Sb(bipy)Cl_3]$, $[PhSb(bipy)Cl_2]$, $[Sb(phen)Cl_3]$, $[PhSb(phen)Cl_2]CH_3COOH$ and $[Sb(dpq)Cl_3]$ was in agreement with the proposed molecular formulas and structures (Figs. 1, 2 and 3). The IR spectra of the complexes in the 1600–400 cm^{-1} region were consistent with coordinated bipy, phen and dpq [36,37]. The polypyridyl ligand bands corresponding to C–C and C–N stretching (1562, 1506, 1422 cm^{-1} for phen; 1578, 1556, 1530 cm^{-1} for bipy; 1582, 1572, 1520 cm^{-1} for dpq) showed considerable changes in the IR spectra of the complexes. Moreover, the strong band of bipy at 758 cm^{-1} with a shoulder at 740 cm^{-1} splitted upon coordination to 772 and 760 cm^{-1} in $[Sb(bipy)Cl_3]$ complex, and to 764 and 742 cm^{-1} in $[PhSb(bipy)Cl_2]$ complex. The 725 cm^{-1} band of phen, assigned to the out of plane motion of the three adjacent hydrogen atoms on the heterocyclic rings, also exhibited marked changes upon the formation of the complexes.

The unequivocal proton NMR assignment was achieved by concerted analysis of 1D ^1H-, 1D ^{13}C-, 2D ^1H ^1H COSY and 2D ^1H ^{13}C-HMQC. The formation of the complexes was also supported by downfield shift in the proton resonance for the protons adjacent to the coordinating nitrogen atoms of all ligands: $\Delta\delta$ 0.06–0.11 ppm in $[Sb(bipy)Cl_3]$, $\Delta\delta$ 0.03–0.06 ppm in $[PhSb(bipy)Cl_2]$, $\Delta\delta$ 0.51–0.59 ppm in $[Sb(phen)Cl_3]$, $\Delta\delta$ 0.31–0.44 ppm in $[PhSb(phen)Cl_2]CH_3COOH$ and $\Delta\delta$ 0.13–0.32 ppm in $[Sb(dpq)Cl_3]$.

3.2. Crystal structures

The structure of $[Sb(phen)Cl_3]$ and $[PhSb(phen)Cl_2]CH_3COOH$ was characterized by X-ray crystallography. The precursor, phenyl antimony(III) dichloride ($PhSbCl_2$) was prepared in acetic acid and used without further purification. The presence of acetic acid in the reaction medium led to cocrystallization of this solvent with $[PhSb(phen)Cl_2]$ complex. Crystal parameters and structure refinement details are summarized in Table 1. The selected bond lengths and angles relevant to the antimony center for complexes $[Sb(phen)Cl_3]$ and $[PhSb(phen)Cl_2]CH_3COOH$ are listed in Tables 2 and 3, respectively.

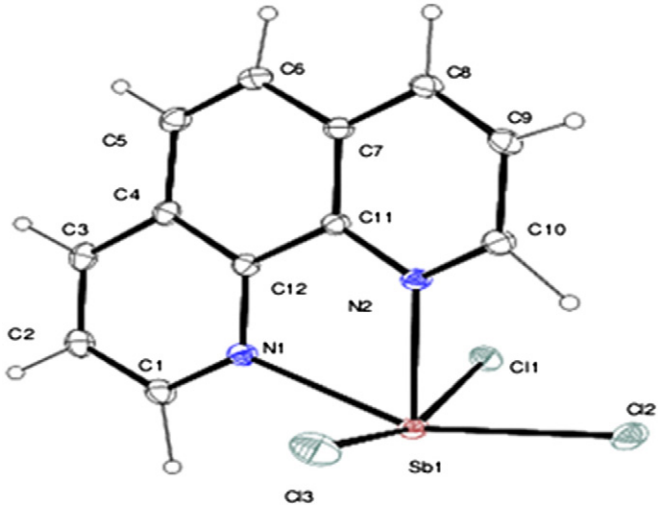

Fig. 2. Molecular structure of $[Sb(phen)Cl_3]$.

The geometry around the metal center, in both complexes, is distorted square pyramidal (SP), with N(2) as the apex and N(1), Cl(1), Cl(2) and Cl(3) occupying the equatorial plane for $[Sb(phen)Cl_3]$ (Fig. 2) and with C(21) as the apex and Cl(1), Cl(2), N(1) and N(2) occupying the equatorial plane for $[PhSb(phen)Cl_2]CH_3COOH$ (Fig. 3).

The root mean square deviation of the plane through atoms Sb, N(1), Cl(1), Cl(2) and Cl(3) of $[Sb(phen)Cl_3]$ is equal to 0.182 Å, with the Sb atom distant 0.3528(4) Å from this plane. This suggests some distortion from an ideal square pyramidal geometry.

In contrast, The root mean square deviation of the plane through atoms Sb, N(1), N(2), Cl(1) and Cl(2) of complex $[PhSb(phen)Cl_2]CH_3COOH$ is equal to 0.069 Å, with the Sb atom being the most distant from the mean plane. The distance from this atom to the plane is equal to 0.1373(8) Å.

From these values, it is possible to conclude that the equatorial coordination of antimony in $[PhSb(phen)Cl_2]CH_3COOH$ is almost planar, in contrast to the coordination in $[Sb(phen)Cl_3]$. The sums of equatorial angles for the two complexes corroborate this conclusion: for $[Sb(phen)Cl_3]$ this sum is equal to 352.95° (Cl(3)–Sb–Cl(2) 95.61 (3)°, Cl(3)–Sb–N(1) 89.78 (5)°, N(1)–Sb–Cl(1) 80.69 (4)°, and Cl(1)–Sb–Cl(2) 86.87 (2)°), while for $[PhSb(phen)Cl_2]CH_3COOH$ the sum is equal to 358.89° (N(1)–Sb(1)–Cl(2) 90.47 (7)°, Cl(2)–Sb(1)–Cl(1) 104.78 (3)°, N(2)–Sb(1)–Cl(1) 94.41 (7)°, and N(2)–Sb(1)–N(1) 69.23 (9)°), suggesting a slight distortion from an ideal square pyramidal [38]. As shown in Fig. 3, the asymmetrical unit in the crystal contains one molecule of acetic acid besides the $[PhSb(phen)Cl_2]$ molecule.

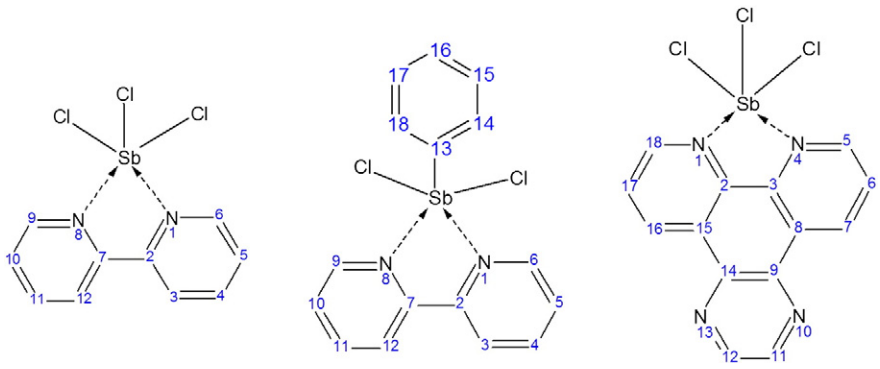

Fig. 1. Molecular structures of $[Sb(bipy)Cl_3]$, $[PhSb(bipy)Cl_2]$ and $[Sb(dpq)Cl_3]$ complexes; ligand atoms are numbered for NMR spectral assignments.

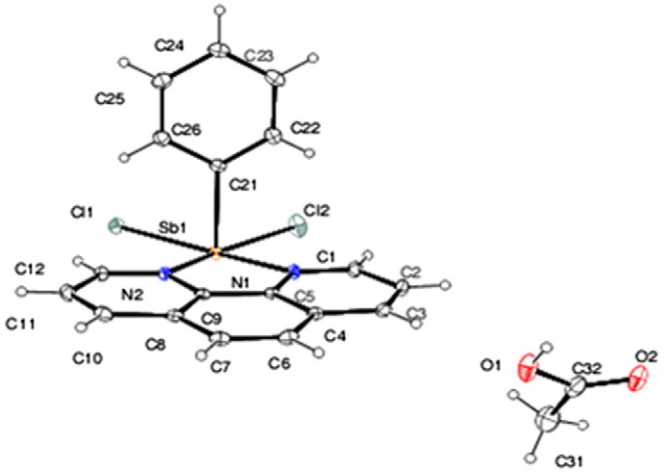

Fig. 3. Molecular structure of $[PhSb(phen)Cl_2]CH_3COOH$.

3.3. Antileishmanial activity of nitrogen donor heterocyclic ligands and their antimony complexes

The ligands, the antimony(III) chlorides and their complexes were evaluated for their activity against Sb^{III}-sensitive and -resistant *Leishmania* strains from two different New World *Leishmania* species: *L. infantum* (syn. *L. chagasi*), the etiological agent for visceral leishmaniasis [39] and *L. amazonensis*, related to the cutaneous form of the disease in the New World [40]. These two species lead to different clinical manifestations, suggesting different metabolism and consequently distinct drug sensitivity. This observation highlights the importance of using different *Leishmania* species in drug screening [41], as well as strains with different susceptibilities to antimonials.

Before starting the biological assays, we first checked the stability of the complexes in water by UV absorption spectroscopy. The UV spectra of the complexes at 40 μM in water were found to differ from those of their respective ligand and did not suffer significant change upon incubation for 30 min at 25 °C (data not shown).

All the antimony complexes were found to be highly active against all *Leishmania* strains with IC_{50} in the 0.25–19 nM range (Table 4). $[PhSb(phen)Cl_2]CH_3COOH$ compound was the most effective complex. Strikingly, the IC_{50} values for Sb complexes were similar when comparing the Sb-resistant mutants with their respective parental sensitive WT strains, except for $[PhSb(phen)Cl_2]CH_3COOH$ and $[Sb(dpq)Cl_3]$ that were 5 and 2.5 times less active in the resistant *L. infantum* strain, respectively. These data are in contrast with that obtained with the conventional Sb^{III} complex potassium antimonyl tartrate which showed IC_{50} greater than 80 μM for WT lines, and was almost inactive against Sb-resistant mutants (Table 4). It is noteworthy that these complexes are much more active than $[Sb(dppz)Cl_3]\cdot H_2O\cdot CH_3OH$ (Sb-dppz complex) [21] and this difference may be due to the less lipophilic character of the actual compounds.

Surprisingly, the ligands and $PhSbCl_2$ also showed very high antileishmanial activities (Table 4), with IC_{50} values in the range of 0.6–22 nM and 0.7–1.8 nM, respectively. The lipophilicity of $PhSbCl_2$ can explain its higher activity, when compared to $SbCl_3$ or TA (Table 4). Another interesting feature is the lack of cross-resistance of $PhSbCl_2$ with $SbCl_3$. These results suggest that $PhSbCl_2$ is not recognized by the membrane transporters involved in the resistance of *Leishmania* to Sb^{III} [6].

To evaluate the impact of antimony complexation of the nitrogen donor ligands on their antileishmanial activity, IC_{50} values were compared between the ligand and its respective complexes (Table 4).

In the case of phen ligand, complexation resulted in enhanced antileishmanial activity, especially with $PhSbCl_2$ compound. The fact that both $PhSbCl_2$ and phen showed IC_{50} in the nanomolar range suggests that both phen and the metal contributed to the overall activity of the complex. On the other hand, the activity of $[Sb(phen)Cl_3]$ may be attributed mainly to the ligand.

In the case of dpq ligand, only small changes in antileishmanial activity were observed upon complexation with antimony. A slight increase of activity was observed only in *L. amazonensis* strains following complexation with $PhSbCl_2$. Thus, it is suggested that essentially the ligand may contribute to the activity of dpq complexes with $SbCl_3$.

In the case of the less active bipy ligand, a marked increase of activity was observed in the *L. infantum* strains following complexation with both metal forms and, in the *L. amazonensis* strains, following complexation with $PhSbCl_2$ only. This data supports the model that both bipy and the metal contributed to the activity of the complex. It also suggests that *L. amazonensis* and *L. infantum* species exhibit differences in their drug metabolism.

Thus, our data strongly suggest that $PhSbCl_2$-derived complexes exert their cytotoxicity through both the ligand and the metal. On the other hand, the mode of action of $SbCl_3$-derived complexes is less clear, since the ligands are cytotoxic in the same concentration range as the complexes and are much more active than $SbCl_3$ or TA. Overall the ligands bipy, phen and dpq are small, planar and non-polar molecules that can readily cross the *Leishmania* cell membrane and form complexes with intracellular transition metal ions triggering their intrinsic antileishmanial effect reported here. In the case of $[Sb(bipy)Cl_3]$ that is more active than bipy, the ligand could act as a Sb carrier facilitating its delivery, leading to a synergistic effect of both ligand and metal against the parasite. However, the lack of cross-resistance of $[Sb(bipy)Cl_3]$ with $SbCl_3$ or TA, i.e. $[Sb(bipy)Cl_3]$ is equally effective against Sb-

Table 4
Growth inhibitory concentrations of the nitrogen donor heterocyclic ligands and their Sb^{III} complexes towards antimony-sensitive and -resistant New World *Leishmania* species.

Compound	[a]IC_{50} ± SEM (nM)			
	L. infantum	*L. infantum*	*L. amazonensis*	*L. amazonensis*
	SbS	SbR	SbS	SbR
$[Sb(phen)Cl_3]$	1.66 ± 0.36	1.93 ± 0.28	0.78 ± 0.18	0.47 ± 0.22
$[PhSb(phen)Cl_2]CH_3COOH$	0.26 ± 0.12	1.26 ± 0.32	0.47 ± 0.08	0.28 ± 0.04
$[Sb(bipy)Cl_3]$	1.34 ± 0.23	1.87 ± 0.14	18.9 ± 2.0	9.1 ± 2.4
$[PhSb(bipy)Cl_2]$	2.21 ± 0.28	2.54 ± 0.13	1.69 ± 0.14	1.65 ± 0.30
$[Sb(dpq)Cl_3]$	0.43 ± 0.10	1.15 ± 0.32	2.28 ± 0.19	1.62 ± 0.28
Phen	7.8 ± 2.4	2.29 ± 0.49	0.84 ± 0.13	0.49 ± 0.08
Bipy	15.8 ± 1.5	21.7 ± 2.7	16.2 ± 1.6	7.4 ± 1.8
Dpq	0.69 ± 0.13	0.85 ± 0.23	2.23 ± 0.20	1.52 ± 0.40
$SbCl_3$	$(341 \pm 2) \times 10^3$	$(462 \pm 3) \times 10^3$	$(362 \pm 2) \times 10^3$	$(1502 \pm 2) \times 10^3$
$PhSbCl_2$	1.79 ± 0.56	1.48 ± 0.08	0.97 ± 0.07	0.74 ± 0.07
[b]TA	$(86 \pm 2) \times 10^3$	$>3000 \times 10^3$	$(110 \pm 1) \times 10^3$	$>3000 \times 10^3$

[a] IC_{50}, concentration of the compound that inhibits 50% of parasite growth; SEM, standard error of the mean. SbS: Sb-sensitive; SbR: Sb-resistant.
[b] TA: potassium antimonyl tartrate as Sb^{III} source.

Table 5
Cytotoxicity of the nitrogen donor heterocyclic ligands and their Sb^{III} complexes against mouse peritoneal macrophages and resulting selective index.

Compound	[a]CC_{50} (nM) macrophages	[b]SI (*L. infantum*)	[b]SI (*L. amazonensis*)
$[Sb(phen)Cl_3]$	12.4 ± 1.2	7.5	16
$[PhSb(phen)Cl_2]$ CH_3COOH	0.91 ± 0.10	3.5	1.9
$[Sb(bipy)Cl_3]$	38.3 ± 1.1	28.6	2.0
$[PhSb(bipy)Cl_2]$	2.11 ± 0.14	0.95	1.2
$[Sb(dpq)Cl_3]$	30.1 ± 3.2	70	13.2
Phen	37.4 ± 3.9	4.8	44
Bipy	$>20 \times 10^3$	>1265	>1234
Dpq	$>8 \times 10^3$	>11,594	>3587
$PhSbCl_2$	4.29 ± 0.36	2.4	4.4
TA	$(63 \pm 1)x10^3$	0.73	0.57

[a] CC_{50}: concentration of the compound that kills 50% of macrophages according to MTT assay.
[b] SI: selective index, calculated as the ratio between CC_{50} in murine macrophages and IC_{50} in the WT *Leishmania* strains.

sensitive and -resistant strains, argues against a cytotoxic action of the metal as $Sb(OH)_3$ which is recognized by the cellular mechanisms of resistance. As alternative explanations for the high activity of $[Sb(bipy)Cl_3]$, the complex may act as a new chemical entity or the metal may improve the bioavailability of the ligand through for instance the increase of its stability in the biological medium.

3.4. Cytotoxicity of nitrogen donor heterocyclic ligands and their antimony complexes against murine peritoneal macrophages

The antimony complexes and their ligand were also evaluated for their cytotoxicity against macrophages, which is one of the host mammalian cell in which *Leishmania* parasite resides, in order to determine their parasite-to-host selectivity index (SI).

When compared to their respective ligand, all complexes showed an enhanced cytotoxicity, the effect being much more pronounced in the case of bipy and dpq ligands which were not cytotoxic in the concentration range tested (Table 5). The Sb^{III} complexes displayed nanomolar cytotoxic concentrations, with CC_{50} values in the range of 0.9–2.1 nM and 12–39 nM for the complexes obtained from $PhSbCl_2$ and $SbCl_3$, respectively. Considering the high cytotoxicity of $PhSbCl_2$ (CC_{50} ~ 4 nM), this metal species was most probably responsible for the toxicity of derived complexes. The increase of cytotoxicity in the case of the complexes formed from $SbCl_3$, to a much higher extent than that of potassium antimonyl tartrate, suggests that the lipophilic complex may enhance the delivery of toxic Sb^{III} to macrophages (compared to hydrophilic tartaric acid) or that these new chemical entities may be more toxic by themselves. The lipophilicity conferred by the phenyl group (Ph) in the case of the complexes derived from $PhSbCl_2$, may also contribute to the increase in cytotoxicity when compared to those obtained from $SbCl_3$or TA (Table 5).

As a consequence of their lower cytotoxicity, the complexes obtained from $SbCl_3$ were found to be more selective than those made from $PhSbCl_2$. In that sense, $[Sb(dpq)Cl_3]$ and $[Sb(phen)Cl_3]$ with SI in the range of 7–70 appear as the most promising compounds, being much more selective than potassium antimonyl tartrate with a SI less than 1 (Table 5).

Another unexpected result emerging from this study is the extremely high selectivity indexes of dpq and bipy ligands, revealing that these compounds are also promising drug candidates by themselves for treatment of Sb-resistant leishmaniasis.

4. Conclusions

Novel trivalent antimony complexes with the nitrogen donor heterocyclic ligand bipy, phen or dpq have been synthesized by the reaction with $SbCl_3$ or $PhSbCl_2$. The crystal structures of $[Sb(phen)Cl_3]$ complex and $[PhSb(phen)Cl_2]CH_3COOH$ organometallic complex showed a distorted square pyramid geometry with a five-coordinated Sb center. All the complexes, the ligands and $PhSbCl_2$ showed very high antileishmanial activities, with IC_{50} in the nanomolar range against Sb^{III}-sensitive and -resistant *L. infantum* (syn. *L. chagasi*) and *L. amazonensis* strains. These compounds were much more active against these *Leishmania* strains than potassium antimonyl tartrate. In the case of the complexes with $PhSbCl_2$, both the ligand and the metal contributed to the overall activity of the complex. Among the complexes formed from $SbCl_3$, only bipy showed an improved activity upon complexation. $PhSbCl_2$ emerges as a new highly active form of Sb^{III} capable of bypassing the resistance mechanisms of *Leishmania* to antimony. However, progress towards new antimonial drugs still depends on the achievement of reduction of the cytotoxicity of the metal against the host mammalian cells.

Abbreviations

RPMI	Roswell Park Memorial Institute (cell culture medium)
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
alpha-MEM	minimum essential culture medium
phen	1,10-phenanthroline
dpq	dipyrido[3,2-d:2′,3′-f]quinoxaline
bipy	2,2′-bipyridine
dppz	dipyrido[3,2-a:2′,3′-c]phenazine
PBS	phosphate-buffered saline

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jinorgbio.2013.12.001.

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